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INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Gordon N.	Gill	La Jolla, CA
Michele	Yeo	La Jolla, CA
Patrick S.	Lin	Davis, CA
Michael E.	Dahmus	Davis, CA

[] Additional inventors are being named on the 0 separately numbered sheets attached hereto.

TITLE OF THE INVENTION (280 characters max)

Phosphatase Regulation of Nucleic Acid Transcription

CORRESPONDENCE ADDRESS

Direct all correspondence to:

[X] Customer Number: 20985

OR

[X] Firm *or* Individual Name	Fish & Richardson P.C.				
Address	4350 La Jolla Village Drive, Suite 500, San Diego, CA 92122				
Country	United States	Telephone	858-678-5070	Fax	858-678-5099

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Respectfully submitted,

Signature

Name Michael Reed, Reg. No. 45,647

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Telephone No. (858) 678-5070

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PROVISIONAL APPLICATION FOR PATENT

under

37 CFR §1.53(c)

TITLE: PHOSPHATASE REGULATION OF NUCLEIC ACID TRANSCRIPTION

APPLICANT: GORDON N. GILL, MICHELE YEO, PATRICK S. LIN AND MICHAEL E. DAHMUS

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Phosphatase Regulation of Nucleic Acid Transcription

Background

[0001] The regulation of cellular gene expression occurs primarily at the level of transcription initiation by RNA polymerase. Regulated transcription initiation by RNA polymerase II in higher eukaryotes involves the formation of a complex with general transcription factors at promoters.

[0002] The largest subunit of RNA polymerase (RNAP) II contains a C-terminal domain (CTD) comprised of multiple repeats of the consensus sequence $Tyr^1Ser^2Pro^3Thr^4Ser^5Pro^6Ser^7$. The progression of RNAP II through the transcription cycle is regulated by both the state of CTD phosphorylation and the specific site of phosphorylation within the consensus repeat (1,2). The emerging overview of this process is that unphosphorylated RNAP II, designated RNAP IIA, enters the preinitiation complex where phosphorylation of Ser 5 is catalyzed by TFIIH (which contains cdk7/cyclin H subunits) concomitant with transcript initiation (3,4). This generates the phosphorylated form of RNAP II, designated RNAP IIO. Ser 5 phosphorylation facilitates the recruitment of the 7-methyl G capping enzyme complex (5-9). Phosphorylation of Ser 2, is catalyzed by the cyclin-

dependent kinase P-TEFb (which contains cdk9/cyclin T subunits)(10,11). Although Ser 5 phosphorylation precedes Ser 2 phosphorylation (12), it is not clear if the dephosphorylation of Ser 5 precedes Ser 2 phosphorylation. During transcript elongation in yeast there is extensive turnover of Ser 2 phosphates mediated by FCP1 and Ctk1, the putative PTEFb homolog (13). Finally, dephosphorylation of Ser 2 by the FCP1 phosphatase regenerates RNAP IIA thereby completing the cycle (14).

[0003] FCP1 is a class C (PPM) phosphatase containing a BRCT domain that is required for interaction with RNAP II and dephosphorylation of the CTD (15,16). FCP1 interacts with and is stimulated by RAP74, the larger subunit of TFIIF (16,17). Class C phosphatases are resistant to inhibitors that block other classes of Ser/Thr phosphatases and bind Mg^{2+} or Mn^{2+} in the binuclear metal center of the catalytic site (18,19). The ψψψDXDX(T/V)ψψ motif (where ψ= hydrophobic residue) present in the FCP1 homology domain characterizes a subfamily of class C phosphatases with both Asp residues being essential for activity (20).

[0004] Synthetic lethality is observed between mutant FCP1 and reduced levels of RNAP II in *S.cerevisiae* and *S. pombe*, indicating that FCP1 is an essential gene (20,21). It remains uncertain whether the activity of FCP1 accounts for

the dephosphorylation of both Ser 5 and Ser 2 and whether this is the sole activity that catalyzes CTD dephosphorylation. Mutations in FCP1 lead to increased phosphorylation of Ser 2 suggesting that it functions *in vivo* in the dephosphorylation of Ser 2 (13). Yeast FCP1 appears specific for Ser 2 phosphate when synthetic peptides are used as substrate (22). However, mammalian FCP1 dephosphorylates both Ser 2 and Ser 5 *in vitro* in the context of native RNAP II (23). Furthermore, modifications such as that catalyzed by the Ess1/Pin1 peptidyl prolyl isomerase may alter the activity and specificity of FCP1 (24-26). Given the importance of CTD phosphorylation in gene expression (27), it is essential to know if additional CTD phosphatases exist and if so, their specificity and the mechanisms by which they are targeted to RNAP II at discrete stages of the transcription cycle.

[0005] Although FCP1 is the only reported CTD phosphatase, examination of the databases reveals additional genes that consist principally of a domain with homology to the CTD phosphatase domain of FCP1. Three closely related human genes encoding small proteins with CTD phosphatase domain homology, but lacking a BRCT domain, have been identified. In the present study we show that a gene located on chromosome 2 encodes a nuclear CTD phosphatase. This

protein preferentially dephosphorylates Ser 5 within the CTD of RNAP II and is stimulated by RAP74. Expression of this small CTD phosphatase (SCP1) inhibits activated transcription from a variety of promoter-reporter gene constructs whereas expression of a mutant lacking phosphatase activity enhances transcription. This newly identified small CTD phosphatase appears to play an important role in the regulation of RNAP II transcription.

Detailed Description

[0006] The invention relates to methods of modifying gene transcription by modulating the activity of small CTD phopsphatases (SCP's). The invention includes methods for identifying compounds that bind to, or interact with, one or more SCP proteins or the DNA/RNA encoding the SCP proteins and, thus, modifying the activity of an SCP protein on RNA polymerase II, or on other transcription factors essential to gene transcription. Compounds that bind to, or interact with, one, or more SCP proteins or the DNA/RNA encoding the proteins can inhibit or enhance the activity of the RNA polymerase II, thus, inhibiting or enhancing gene transcription. For example, antisense, nonsense or interfering (i.e., RNAi) nucleotide sequences

that modulate SCP translation or transcription can effect the RNA polymerase II-mediated gene transcription.

[0007] The invention further relates to methods of identifying compounds that modulate SCP activity by in vitro transcription. This invention further relates to methods of identifying substances that modify gene transcription, and methods of treating disease conditions resulting from insufficient, increased or aberrant production of SCP proteins. These methods include the use of substances that bind to, or interact with, the SCP proteins, (naturally occurring and biologically active, also referred to herein as wildtype SCP proteins) genes encoding the SCP proteins, SCP messenger RNA, or the use of genetically altered SCP proteins.

[0008] Compounds used in the methods described herein can be proteinaceous in nature, such as peptides (comprised of natural and non-natural amino acids) and peptide analogs (comprised of peptide and non-peptide components), or can be non-proteinaceous in nature, such as small organic molecules. The substance can also be a genetically engineered SCP protein with an altered amino acid sequence. These substances would be designed to bind to, or interact with the SCP protein based on the DNA or amino acid

sequences of the SCP proteins described herein, or antibodies reactive with the SCP proteins described herein.

[0009] For example, a substance can be identified, or designed, that specifically interferes with the phosphatase activity of one, or more, SCP proteins thereby inhibiting RNA polymerase II holoenzyme activity. Monoclonal or polyclonal antibodies (e.g, the polyclonal antibodies described herein) specific for one, or more, of the SCP proteins can also be used to prevent, or inhibit, the SCP proteins from participating in the initiation of gene transcription.

[0010] The invention provides methods (also referred to herein as a "screening assays") for identifying modulators, i.e., candidate or test compounds or agents (e.g., peptides, peptidomimetics, small molecules or other drugs) which bind to SCP proteins, have a stimulatory or inhibitory effect on, for example, SCP expression or SCP activity, or have a stimulatory or inhibitory effect on, for example, the activity of an SCP target molecule.

[0011] In one embodiment, the invention provides assays for screening candidate or test compounds which are target molecules of a SCP protein or polypeptide or biologically active portion thereof. In another embodiment, the invention provides assays for screening candidate or test

compounds which bind to or modulate the activity of a SCP protein or polypeptide or biologically active portion thereof. The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) Anticancer Drug Des. 12:145).

[0012] In one embodiment, an assay is a cell-based assay in which a cell which expresses a SCP protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to modulate SCP activity determined. Determining the ability of the test compound to modulate SCP activity can be accomplished by monitoring the bioactivity (i.e., phosphatase activity) of the SCP protein or biologically active portion thereof. The cell, for example, can be of mammalian origin.

[0013] SCP1 and SCP2 were obtained as EST clones from Resgen. The full-length cDNA for SCP1 (261 aa, BE300370), the cDNA encoding the spliced variant of SCP1 (214 aa, AL520011) and SCP2 (AL520463) were subcloned into EcoR1-Xho1 sites of pGEX4T-1 and pcDNA3Flag vectors by PCR. The D96E, D98N mutant of SCP1 261 and the corresponding mutant of SCP1 214, D48E and D50N, were generated by QuikChange (Stratagene). All constructs were verified by sequencing. GST fusions were purified by glutathione-sepharose chromatography and SCP1 261 was generated by cleavage at the thrombin site encoded in the vector. Recombinant FCP1 was expressed and purified as described previously (23).

[0014] Human recombinant casein kinase II (CKII) and mouse recombinant MAPK2/ERK2 were obtained from Upstate Biotechnology. Human CTDK1/CTDK2 were purified as described by Payne and Dahmus (28). Human TFIIH was obtained as described (29). Human P-TEFb was partially purified from HeLa S-100 extract by chromatography on Heparin-Sepharose (Amersham Biosciences), DEAE 15HR (Millipore) HiTrap S and Phenyl-Superose (both from Amersham Biosciences). P-TEFb was dialyzed against 25 mM Hepes, pH 7.9, 20% glycerol, 25 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF. Human recombinant Cdc2 kinase was purchased from New England Biolabs. Rabbit anti-SCP1 IgG was prepared by ammonium sulfate

fractionation and protein G-sepharose chromatography. RNAP II antibodies (8WG16, H5 and H14) were obtained from Covance.

[0015] *Preparation and Purification of ^{32}P-RNAP IIO Isozymes and ^{32}P-GST-CTDo*: Calf thymus RNAP IIA was purified by the method of Hodo and Blatti (30) with modifications as described by Kang and Dahmus (31). Specific isozymes of ^{32}P-labeled RNAP IIO were prepared by phosphorylation at the most C-terminal serine (CKII site) in the largest subunit of purified RNAP IIA with recombinant CKII and [γ-^{32}P]ATP, followed by CTD phosphorylation in the presence of 2 mM ATP with either purified CTDK1/CTDK2, TFIIH, P-TEFb, recombinant MAPK2/ERK2 or recombinant Cdc2 kinase. The RNAP IIO isozymes were individually purified over a DE53 column with a step elution of 500 mM KCl (28). Because only the most C-terminal serine is labeled with ^{32}P and lies outside the consensus repeat, dephosphorylation by CTD phosphatase results in an electrophoretic mobility shift in SDS-PAGE of subunit IIo to the position of subunit IIa without the loss of label. Similarly, ^{32}P-labeled GST-CTDo was prepared from GST-CTDa by CKII followed by MAPK2/ERK2. GST-CTDo was purified over a glutathione-agarose column with a step elution of 15 mM glutathione.

[0016] *Phosphatase Assays*: PNθP reaction mixtures (200 µl) containing 50 mM Tris-acetate, pH 5.5, 10 mM $MgCl_2$, 0.5 mM DTT, 10% glycerol, 20 mM PNθP and recombinant proteins were incubated for at 30°C for 1 hr. The reactions were quenched by adding 800 µl of 0.25 N NaOH. Release of pNθ was determined by measuring A_{410}.

[0017] N-terminal biotinylated CTD phosphopeptides, comprised of 4 tandem repeats YSPTSPS and containing phosphoserine at position 2 or position 5, were synthesized (*Alpha Diagnostics*, San Antonio, TX). Phosphatase reaction mixtures (50 µl) containing 50 mM Tris-acetate, pH 5.5, 10 mM $MgCl_2$, 0.5 mM DTT, 10% glycerol, 25 µM of phosphopeptide and wild type or mutant SCP1 were incubated for 60 mins at 37°C. The reactions were quenched by adding 0.5 ml of malachite green (Biomol). Phosphate release was measured at A_{620} and quantifiedrelative to a phosphate standard curve.

[0018] CTD phosphatase assays utilizing RNAP IIO and GST-CTDo as substrate were performed as described previously (32) with minor modifications. Reactions were performed in 20 µl of CTD phosphatase buffer (50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 20% glycerol, 0.025% Tween 80, 0.1mM EDTA, 5 mM DTT) in the presence of 20 mM KCl. Each reaction contained specified amounts of GST-CTDo and/or RNAP IIO and was carried out in the presence of 7 pmol RAP74. Reactions were

initiated by the addition of FCP1 or SCP1 and incubated at 30°C for 30 minutes. Assays were terminated by the addition of 5X Laemmli buffer, and RNAP II subunits and GST-CTD were resolved on a 5% SDS-PAGE gel. The gel images were developed by autoradiography and scanned by Molecular Dynamics Image Scanner Storm 860 in the phosphor screen mode. Data were quantitatively analyzed by ImageQuant software.

[0019] *Tissue Culture and Transfections*—Human 293, COS7 and CV1 cells were grown at 37°C in DMEM supplemented with 10% normal calf serum (BRL). Subconfluent cells were transfected in 6 well tissue culture dishes using Effectene (Qiagen) according to the manufacturer's instructions. Reporter and activator plasmids (100 ng each) and Flag SCP1 (80 ng) or its mutant were used per well. For T3 and PPARγ transfections, 20 ng RXR plasmid was also added. The amount of ligands used are as follows: 100 nM T3, 1 μM PPARγ609843 (Ligand Pharmaceuticals), 100 nM dexamethasone. LMX1 and E47 expression plasmids (100 ng) were cotransfected with 100 ng of the rat insulin promoter-luciferase reporter construct with the indicated concentrations of SCP1 and phosphatase-minus SCP1 expression plasmids. The total amounts of transfected DNA was kept constant by the addition of empty vector. Cells were harvested 48 hrs after

transfections and cellular extracts were assayed for luciferase activity using Luciferase Assay System (Promega) according to the manufacturer's instructions.

[0020] *Immunofluorescence*: Cells grown on coverslip were fixed in 2% paraformaldehyde, neutralized and blocked using 2.5% FCS/PBS. Rabbit polyclonal IgG 6703 was used at 1:100 dilution, followed by goat anti-rabbit IgG H+L chains conjugated to Alexa Fluor 488 (1:250). Mouse anti EEA1 was used at 1:1000 followed by goat anti-mouse IgG conjugated to Alexa Fluor 594 (1:250). Omission of primary antibodies was used as negative control. The coverslips were viewed using the Zeiss Axiophot which is equipped with a Hamamatsu Orca ER firewire camera that runs on Improvision Openlab 3.0.9 software.

[0021] *Immunoprecipitations*: For immunoprecipitation experiments, 75% confluent COS7 cells from a 10 cm dish were harvested in lysis buffer (PBS containing 1% NP40, 1 mM DTT and protease inhibitors). Lysates were incubated with 20 µl sepharose-conjugated anti-SCP1 (6703) IgG at 4°C for 6 hr. Beads were washed with PBS and the complexes were evaluated by western blotting using specific anti-RNAP II antibodies. Rabbit anti-SNX1 antibody was used as control IgG.

[0022] The alignment of three human proteins that are closely related to one another and have homology to the phosphatase domain of human FCP1 is shown in Figure 1A. All contain the signature motif ΨΨΨDXDX(T/V)ΨΨ. SCP1, located on chromosome 2q35, was initially designated nuclear LIM interacting factor (NLI-IF) in the genome entry (33). The full-length 261 aa protein is encoded by 7 exons; a shorter NH_2 terminal splice version of 214 aa is present in EST databases. SCP1 has ~20% homology to human FCP1 in the phosphatase domain while the 3 SCP proteins are >90% homologous in this region. SCP2/OS4 located on chromosome 12q13 was co-amplified with CDK4 in sarcomas (34) and SCP3/HYA22 located on chromosome 3q22 was part of a large chromosome deletion in a lung carcinoma cell line (35). These represent a subset of proteins with putative CTD phosphatase-like catalytic domains found in plants, yeast, nematodes and arthropods. The Drosophila and Anopheles genomes each contain a single highly conserved SCP ortholog. The SCP proteins lack the BRCT domain present in FCP1 (Figure 1B).

[0023] To determine whether SCP1 has phosphatase activity, the protein was expressed as a GST-fusion and both SCP1 261 and SCP1 214 were assayed using PNθP as substrate. As

reported for FCP1 from *S. pombe* (22) utilizing PNθP as substrate, the pH optimum for SCP1 phosphatase activity is near 5 (Figure 2A). Phosphatase activity was Mg^{2+}-dependent and resistant to the phosphatase inhibitors okadaic acid and microcystin (Figure 2B). Ca^{2+} could not substitute for Mg^{2+}. Mutations of Asp96 to Glu (D96E) had little to no effect on phosphatase activity (data not shown) whereas mutating Asp98 to Asn (D98N) in conjunction with the D96E mutation completely abolished phosphatase activity (Figure 2B). SCP1 is thus a class 2C phosphatase whose activity is dependent on acidic residues in the conserved DXD motif. SCP2 exhibited similar phosphatase activity (Figure 2B).

[0024] To determine whether GST-SCP1 214 has CTD phosphatase activity, GST-CTDo and RNAP IIO were utilized as substrates and the activity of SCP1 was compared directly with that of FCP1. Recombinant CTDo (rCTDo) and RNAP IIO utilized as substrate in these experiments were prepared by the phosphorylation of purified GST-CTDa or RNAP IIA with casein kinase II (CKII) in the presence of [γ-^{32}P]ATP followed by phosphorylation with MAPK2/ERK2 in the presence of excess unlabeled ATP. MAPK2/ERK2 was used in these initial experiments because it phosphorylates both GST-CTDa and RNAP IIA with comparable efficiency (23). As

expected, FCP1 efficiently converts RNAP IIO to RNAP IIA in a processive manner (Figure 2C, lanes 7-12) (23,32). Even high concentrations of FCP1 did not result in measurable dephosphorylation of rCTDo (Figure 2C, lanes 1-6) consistent with the idea that a docking site on RNAP II is required for activity (17). GST-SCP1 214 catalyzed the dephosphorylation of both RNAP IIO and GST-CTDo with comparable efficiency (Figure 2D). In contrast to FCP1, the SCP1 catalyzed dephosphorylation of RNAP IIO appears non-processive in that a number of phosphorylated intermediates are visible in SDS-PAGE. SCP1 is specific for dephosphorylation of the consensus repeat in that the phosphate at the CKII site is not removed. As shown below, the pattern of dephosphorylation varies depending on the CTD kinase used in the preparation of RNAP IIO. Mutant SCP1 (D96E, D98N) lacked activity on either substrate (data not shown). SCP1 is thus a CTD phosphatase that acts on both RNAP IIO and rCTDo. SCP2 exhibits comparable CTD phosphatase activity when RNAP IIO is utilized as substrate (see Figure 4).

SCP1 preferentially dephosphorylates Ser 5 of the CTD heptad repeat

[0025] To determine the specificity of SCP1 with respect to its ability to dephosphorylate specific positions within the consensus repeat, RNAP IIO isozymes were prepared *in vitro* by the phosphorylation of RNAP IIA with CTD kinases of known specificity. TFIIH, P-TEFb and MAPK2/ERK2 preferentially phosphorylate Ser 5 when synthetic peptides serve as substrate (36,37) whereas Cdc2 kinase phosphorylates Ser 2 and Ser 5 (38). Although the specificity appears relaxed when RNAP II serves as substrate, RNAP IIO prepared with Cdc2 kinase is clearly distinct from RNAP IIO generated by other CTD kinases (23). Results presented in Figure 3A indicate that RNAP IIO, prepared by the phosphorylation of RNAP IIA with distinct CTD kinases, exhibit a differential sensitivity to dephosphorylation with SCP1. SCP1 most efficiently dephosphorylates RNAP IIO generated by TFIIH and was unable to dephosphorylate RNAP IIO prepared with Cdc2 kinase. SCP1 was also unable to dephosphorylate RNAP IIO generated by Abl tyrosine kinase (data not shown). The dephosphorylation of RNAP IIO isozymes prepared with P-TEFb, MAPK2/ERK2 and CTDK1/CTDK2 occurred at a reduced rate relative to that of RNAP IIO prepared with TFIIH. Furthermore, while the

dephosphorylation reaction appears processive for RNAP IIO prepared by TFIIH, it is clearly non-processive for RNAP IIO generated by MAPK2/ERK2. In contrast FCP1 shows no preference for RNAP IIO generated by TFIIH and efficiently dephosphorylates RNAP IIO generated by Cdc2 kinase (23). These results suggest SCP1 differs from FCP1 in substrate specificity, showing relative preference for the dephosphorylation of Ser 5 in the heptad repeat.

[0026] To investigate the relative reactivity of SCP1 for Ser 2 and Ser 5, a synthetic 28 aa peptide containing 4 heptad repeats phosphorylated exclusively on Ser 2 or on Ser 5 was dephosphorylated in the presence of increasing amounts of SCP1. As shown in Figure 3B, SCP1 preferentially dephosphorylates the Ser 5 phosphopeptide compared to the Ser 2 phosphopeptide. This substrate specificity contrasts to that reported for FCP1 from *S. pombe* which referentially dephosphorylate the Ser 2 phosphopeptide (22). Mammalian FCP1, within a comparable concentration range, did not act on either phosphopeptide (data not shown). These results using synthetic phosphopeptide substrates confirm that SCP1 preferentially dephosphorylates Ser 5 phosphate of the CTD.

[redacted]

Effect of RAP74 on the activity of SCP1

[0027] The RAP74 subunit of TFIIF stimulates CTD phosphatase activity of FCP1 (17). Furthermore, the domains of FCP1 that bind RAP74 are required for FCP1-dependent viability in *S. cerevisiae* (15). Therefore, it was of interest to determine if RAP74 can also influence the activity of SCP. CTD phosphatase activity was measured at low enzyme concentrations to more readily detect stimulatory effects of RAP74. As shown in Figure 4, RAP74 shifted the dose response curve for SCP1 catalyzed dephosphorylation of RNAP IIO to an approximately 10-fold lower concentration. The CTD phosphatase activity of the GST-fusion forms of SCP1 261, SCP1 214 and SCP2 were also enhanced by RAP74. In support of the conclusion that RAP74 stimulates the activity of SCPs, RAP74 bound directly to GST-SCP1 but not to GST (data not shown). The binding and stimulatory effects of RAP74 suggest that TFIIF is important for optimal CTD phosphatase activity for both FCP1 and SCP1.

SCP1 is located in the nucleus associated with RNAP II

[0028] Although SCP1 lacks an obvious nuclear localization sequence, it is found in the nucleus. Immunofluorescence microscopy using a rabbit polyclonal anti-SCP1 antibody

demonstrated nuclear localization of endogenous SCP1 in COS7 cells (Figure 5 B). Co-staining with DAPI for nuclear identification and with the early endosomal marker EEA1 for cellular detail confirmed the specific localization of SCP1 in nuclei (Figure 5 panels A and B).

[0029] Co-immunoprecipitation was used to assess the association of SCP1 with RNAP II. Sepharose-immobilized anti-SCP1 IgG 6703 was used to immunoisolate SCP1 from COS7 cells. Immunoisolates were resolved by SDS-PAGE and blotted with anti-RNAP II antibodies. As shown in Figure 5C, RNAP II was present in SCP1 immunoprecipitates indicating that SCP1 and RNAP II either interact directly or are in the same macromolecular complex. To determine whether SCP1 preferentially associated with either Ser 2 or Ser 5 phosphorylated RNAP IIO, lysates were prepared in the presence of EDTA, to inhibit phosphatase activity. SCP1 immunoprecipitates were then blotted with monoclonal antibodies specific for Ser 2 phosphate (H5) and Ser 5 phosphate (H14). Both forms of RNAP IIO were present in COS7 cell lysates. Ser 5 phosphate-enriched RNAP IIO appeared to be preferentially associated with SCP1 in immunoprecipitates as indicated by the ratios of co-immunoprecipitated RNAP IIO relative to the amount of RNAP IIO contained in the extract (Figure 5C).

SCP1 affects RNAP II transcription *in vivo*

[0030] To assess the effect of SCP1 on transcription *in vivo*, the activity of a variety of luciferase reporter gene constructs was examined in the presence or absence of cotransfected SCP1. Targeting a Gal 4-DNA binding domain SCP1 fusion or the phosphatase-inactive Gal 4-SCP1 mutant upstream of a thymidine kinase promoter-luciferase reporter (Gal 4-TK-Luc) had no significant effect on transcriptional activity (Figure 6A). Interestingly, untethered SCP1 in the presence of several reporter constructs had no significant effect on reporter gene expression whereas the inactive mutant resulted in a significant stimulation of expression from the E1ALuc, pGL3-Luc and Gal4-TATA-Luc constructs (Figure 6B). The phosphatase-minus SCP1 mutant increased luciferase activity 1.5- to 6-fold.

[0031] In contrast, WT or phosphatase-inactive SCP1 affected reporter gene expression from a variety of regulated promoters. Luciferase activity from a Gal 4-TK-Luc reporter that was strongly stimulated by co-expressing a Gal 4-VP16 fusion protein (30-fold stimulation) was strongly inhibited by SCP1 (Figure 6C). In contrast, phosphatase-inactive SCP1 enhanced Gal 4-Vp16-stimulated activity about 2-fold.

[0032] Opposing effects of active SCP1 and the inactive mutant SCP1 were observed using a number of inducible promoter-reporter constructs. Ligand-activated T_3 receptor activity on a DR+4 TRE-TK-Luc reporter gene was inhibited by active SCP1 and enhanced by inactive SCP1 (Figure 6D). Similar results were obtained when the C-terminus of $T_3R\beta$ was fused to the Gal 4-DNA binding domain (Gal 4-$T_3R\beta C$) and targeted to Gal 4-TK-Luc. SCP1 also inhibited dexamethasone-stimulated glucocorticoid receptor activity on a GRE-TK-Luc construct whereas mutant SCP1 significantly enhanced activity (Figure 6D). Finally, SCP1 inhibited ligandactivated PPARγ receptor activity assayed on a PPARγ promoter response element and mutant SCP1 enhanced activity (Figure 6D). A similar response pattern was observed when the Gal 4-DNA binding domain was fused to the C-terminus of PPARγ and targeted to Gal 4-TK-Luc (data not shown). The same pattern of responses was observed in HEK293, COS-7 and CV-1 cells(data not shown).

[0033] Although there was variability in the extent of inhibition and stimulation of transcription observed with SCP1 and mutant SCP1, respectively, the overall patterns were similar using additional inducible systems such as estrogen and retinoic acid receptor responsive promoters. The extent of inhibition and stimulation likely results

from variable expression levels of transfected proteins as well as potentially different rate limiting steps in transcription from specific promoters.

[0034] The competing effects of SCP1 and mutant SCP1 were further examined using a rat insulin promoter-luciferase construct. There is strong synergy on this promoter between the bHLH protein E47 and the LIM-homeodomain protein LMX1 which bind to adjacent DNA target sites (39,40). Co-expression of LMX1 and E47 enhanced luciferase activity 25-fold from the rat insulin 1 promoter (Figure 6E). When phosphatase-inactive SCP1 was held constant, increasing amounts of SCP1 inhibited luciferase expression (Figure 6E). In the presence of mutant SCP1, the SCP1 inhibition curve was right-shifted (~20% inhibition with 40 ng SCP1 plasmid plus 20 ng mutant SCP1 plasmid vs. >90% inhibition with 40 ng SCP1 plasmid alone (Figure 6E)). With a constant input of SCP1 plasmid, increasing amounts of mutant SCP1 not only blocked the inhibitory effects of SCP1, but enhanced activity significantly. The stimulatory effects of transfected phosphatase-inactive SCP1 are thus consistent with it acting as a dominant negative.

[0035] Acquisition of the CTD of RNAP II allows extensive protein interactions and is thought to have been an important step in the evolution of complex patterns of

regulated gene expression (41). Most importantly, the CTD can exist in multiple conformations thereby facilitating the recruitment of different multiprotein complexes at specific points in the transcription cycle. Both the site of phosphorylation within the consensus repeat, Ser 2 or Ser 5, and the extent of phosphorylation of the CTD control many aspects of RNAP II transcription, including the recruitment of RNAP II to the preinitiation complex, initiation, capping, elongation, splicing and polyadenylation (27). Ser 2 and Ser 5 within the consensus CTD repeat are essential residues and distinct CTD kinases catalyze phosphorylation at these sites (42). To date, a single CTD phosphatase, FCP1, has been implicated in removing phosphates (for review see (43)). The present study establishes that a related family of proteins, SCPs, have CTD phosphatase activity, are stimulated by RAP74 and modulate gene expression *in vivo*.

[0036] SCP1 preferentially catalyzes dephosphorylation of RNAP IIO phosphorylated by TFIIH. RNAP IIO phosphorylated by P-TEFb and MAPK2/ERK2 are also dephosphorylated by SCP1 but at a reduced rate. RNAP IIO phosphorylated by Cdc2 kinase, which preferentially phosphorylates Ser 2 with some phosphorylation at Ser 5, is not a substrate for SCP1. The preferential dephosphorylation of Ser 5 by SCP1 was

confirmed using a 4 heptad repeat peptide substrate. Interestingly, the specificity of SCP1 contrasts with that of *S. pombe* FCP1 which prefers Ser 2 with a similar peptide substrate (22). However, FCP1 dephosphorylates Ser 2 phosphates and Ser 5 phosphates with comparable efficiency when native RNAP IIO serves as substrate *in vitro* (23). The relative specificity of FCP1 also differs from SCP1 in that unlike FCP1, SCP1 shows preference for the dephosphorylation of TFIIH phosphorylated RNAP IIO.

[0037] It is clear from the results presented in Figure 2C and D that, when RNAP IIO phosphorylated with MAPK2/ERK2 is utilized as substrate, the specific activity of SCP1 is substantially lower than that of FCP1. This difference in specific activity is in part due to the fact that MAPK2/ERK2 phosphorylated RNAP IIO is an especially poor substrate for SCP1 (Figure 3) whereas FCP1 dephosphorylates different isozymes of RNAP IIO with comparable efficiency (23). The amount of SCP1 required to dephosphorylate MAPK2/ERK2 phosphorylated RNAP IIO is 50 to 100 fold higher than that required to dephosphorylate TFIIH phosphorylated RNAP IIO. Furthermore, the BRCT domain in FCP1 that facilitates its interaction with RNAP II is absent from SCP1. Most importantly, without a better understanding of the factors that influence the recruitment of SCP1 to

complexes containing RNAP IIO, it is difficult to interpret differences in specific activity in a binary reaction. The finding that SCP1 dephosphorylates rCTDo and RNAP IIO with comparable efficiency whereas FCP1 does not dephosphorylate rCTDo even at concentrations 100 times higher than that required to dephosphorylate RNAP IIO, suggest that the interaction of SCP1 and FCP1 with the CTD require different molecular interactions.

[0038] During the transcription cycle, protein complexes assemble and disassemble on the CTD in a dynamic and regulated manner. Ser 5 phosphorylation is detected primarily at the promoter region, whereas Ser 2 phosphorylation is seen in coding regions (12). Phosphorylation of Ser 5 facilitates recruitment of the capping enzymes and allosterically activates their activity (5-9,44,45). Given the preference of SCP1 for phosphoserine 5, SCPs are candidates for acting early in the transcription cycle, perhaps facilitating the transition from initiation and capping to promoter clearance and processive transcript elongation.

[0039] Like FCP1, SCP1 phosphatase activity is found in a complex with RNAP II and its activity is stimulated by RAP74. Mapping studies indicate that the C-terminal domain of FCP1 distal to the phosphatase domain interacts with

TFIIF (15,16). A region near the C-terminus of SCP1 shares homology with the putative RAP74 interaction domain in CP1. Additional studies are necessary to establish if this region of SCP1 mediates its interaction with RAP74. These observations suggest that RAP74 plays an important role in regulating phosphate turnover at both Ser 2 and Ser 5 consistent with the involvement of TFIIF in assembling RNAP II into preinitiation complexes and in stimulating the rate of transcript elongation.

[0040] The results of reporter gene assays indicate that over-expression of either WT or mutant SCP1 can influence gene expression. The overexpression of mutant SCP1 activates transcription several fold from nearly all promoters examined (Figure 6B, C and D) whereas overexpression of WT SCP1 appears to selectively inhibit activated transcription from a variety of inducible promoter-reporter gene constructs (Figure 6D). Because mutant SCP1 is competitive with SCP1, the stimulatory effects are consistent with partial inhibition of endogenous SCP. The stimulatory effects of the phosphatase-inactive mutant suggest that the Ser 5 dephosphorylation cycle is highly dynamic and likely carefully regulated. Based on the assumption that mutant SCP1 is recruited to the promoter, overexpression would shift the equilibrium in

the direction of increased Ser 5 phosphorylation. This could in principle lead to an increase in the efficiency of cap formation and promoter clearance. The inhibitory effects of overexpressed SCP1 are consistent with excessive dephosphorylation of the CTD, perhaps leading to a decrease in the efficiency of cap formation, promoter clearance and/or transcript elongation. Because of the close sequence homologies between SCP1, SCP2 and SCP3, mutant SCP1 may interfere with all 3 family members. The basis for the apparent selectivity in inhibiting activated transcription is not clear. This could in principle result from an increased efficiency in the recruitment of SCP1 to a given promoter and/or a difference in the rate limiting step during initiation or promoter clearance.

[0041] The present studies do not exclude the possibility that SCP1 influences the state of phosphorylation of substrates other than the CTD nor do they address the function of related family members which may function in a variety of pathways including those located at the cell surface and nuclear membrane (46,47). SCP1 co-immunoprecipitates with the nuclear LIM interactor of LIM homeodomain proteins (48) as well as with transcription factors such as the T_3 receptor, Lhx1, Mesp1 and Olig2 indicating it is not a specific NLI-IF (data not shown).

SCP2/0S4 was initially identified in a chromosomal region frequently amplified in sarcomas and brain tumors (34). The ectopic expression of the Xenopus ortholog induced mesoderm marker expression and formation of a secondary dorsal axis. Mutation of the DXDX(T/V) motif abrogated the latter effect (49), suggesting that phosphatase activity is essential for duplicate axis formation. In contrast SCP3 /HYA22 was initially identified in a genomic region homozygously deleted in a lung carcinoma cell line (35). The level of expression of each SCP family member may thus affect gene transcription and have overlapping but also diverse biological effects. SCP1, 2 and 3 are widely expressed in human tissues with highest expression observed in skeletal muscle and lowest expression in brain (33,34).

[0042] SCP1 and related Ser 5 phosphatases may thus complement FCP1 to control the orderly cycle of phosphorylation and dephosphorylation of the CTD during the transcription cycle. An understanding of the precise role that specific CTD phosphatases play in the transcription cycle is dependent on defining the specific point in the cycle at which they act and the mechanism by which they are recruited to RNAP II containing complexes. Given the importance of the CTD in mediating the synthesis and processing of the primary transcript, an understanding of

the dynamics of CTD phosphorylation is central to elucidating the mechanisms and regulation of gene expression.

Abbreviations

[0043] CKII, casein kinase II; CTD, C-terminal domain of RNA polymerase II; FCP1, TFIIF associated CTD phosphatase; NLI-IF, nuclear LIM interacting factor; PNθP, para-nitrophenylphosphate; PP2C, protein phosphatase 2C; P-TEFb, positive transcription elongation factor b; RAP74, RNA polymerase II associated protein of 74 kDa (larger subunit of TFIIF); RNAP, RNA polymerase; SCP, small CTD phosphatase.

Brief description of the drawings

[0044] Figure 1. Alignment of sequences surrounding the catalytic domain and relation of SCP to FCP1

A. Sequence alignment and relationship of 3 small phosphatases with FCP1. Bracket indicates the conserved signature motif and * indicate critical Asp residues involved in phosphatase activity. Previous descriptive names and chromosome locations are indicated. Multiple alignments were done using Clustal W algorithm with Vector NTI Suite (Informax).

B. Domain structures of FCP1 and SCP proteins.

[0045] Figure 2. SCP1 is a class C CTD phosphatase

A. pH optimum for SCP1 utilizing synthetic peptide substrates. GST-SCP1 214 (40 pmol) was incubated with 20 mM PNθP for 60 min at 30°C and phosphatase activity measured by the change in A_{410}.

B. Divalent metal ion requirement for SCP1 activity. The phosphatase activity of GST-SCP1 214 (40 pmol) was measured in the presence of 20 mM PNθP and varying concentrations of $[Mg^{2+}]$ or $[Ca^{2+}]$. Activity was also measured in the presence of 1-10 μM okadaic acid and 1-10 μM microcystin. The 10μM concentration is shown. Mutant SCP1 (D96E D98N) was inactive (- - -). SCP2 also exhibited phosphatase activity (□).

C. and D. CTD phosphatase assay of FCP1 and SCP1 on GST-CTDo and RNAP IIO prepared by MAPK2/ERK2. Increasing amounts of FCP1 or GST-SCP1 214 were assayed in the presence of 75 fmol GST-CTDo (lanes 1-6), 75 fmol RNAP IIO (lanes 7-12), or 75 fmol GST-CTDo and 75 fmol RNAP IIO (lanes 13-18). Both GST-CTDo and RNAP IIO substrates were prepared by the *in vitro* phosphorylation of CKII-labeled GST-CTDa or RNAP IIA by MAPK2/ERK2. All reactions were carried out in the presence of 7 pmol RAP74. CTD dephosphorylation of both GST-CTDo and RNAP IIO is shown by

the increase in mobility of GST-CTDo to GST-CTDa and subunit IIo to IIa, respectively. The difference in the intensity of radiolabeled GST-CTDoand that of radiolabeled subunit IIo is not a reflection of a difference in the amount of substrates present, but of the higher efficiency with which CKII incorporates radiolabeled phosphates onto the most C-terminal serine of GST-CTDa compared to subunit IIa.

[0046] Figure 3. Substrate specificity of SCP1.

A. Dephosphorylation of RNAP IIO prepared with various CTD kinases. Increasing amounts of GST-SCP1 214 were assayed in the presence of 3.7 fmol RNAP IIO prepared by CTDK1/CTDK2, TFIIH, P-TEFb, MAPK2/ERK2 and Cdc2 kinase. All reactions were carried out in the presence of 7 pmol RAP74. CTD dephosphorylation of RNAP IIO isozymes by GSTSCP1 214 is shown by an increase in mobility of subunit IIo to that of IIa. The results are summarized in the graph showing the percent of RNAP IIO remaining as a function of increasing SCP1 concentrations.

B. Effects of GST-SCP1 214 on a 28 aa peptide consisting of heptad repeats containing either Ser 5 phosphate or Ser 2 phosphate. The indicated amounts of SCP1 were incubated with the phosphopeptide substrate and phosphate released was measured as described under "Experimental Procedures."

[0047] **Figure 4. Effect of RAP74 on CTD phosphatase activity of SCP1 and SCP2.** Increasing amounts of the indicated forms of SCP1 and SCP2 were assayed in the presence of 14.4 fmol RNAP IIO prepared by TFIIH. Reactions were carried out in the presence and in the absence of 7 pmol RAP74.

[0048] **Figure 5. Nuclear localization and association of SCP1 with RNAP II.**

A and B. Cells were co-stained for the endosomal marker EEA1 using mouse anti-EEA1 and Alexa Fluor 594 conjugated goat anti-mouse (red). Nuclei were detected with DAPI (blue) (panel A). Immunofluorescence microscopy detection of endogenous SCP1 using rabbit polyclonal IgG 6307 and Alexa Fluor 488 conjugated goat anti-rabbit IgG (green) (panel B). Second antibodies alone served as a control in all cases.

C. Coimmunoprecipitation of RNAP II and endogenous SCP1. Extracts from untransfected COS7 cells were immunoprecipitated using sepharose-immobilized anti-SCP1 IgG 6703 or sepharose-immobilized control IgG. Immunoprecipitates were resolved on SDS-PAGE and blotted using anti-RNAP II antibody (8WG16) and with the Ser 2 phosphate epitope specific antibody H5 and the Ser 5 phosphate epitope specific antibody H14. RNAP IIO present

in COS7 lysates is shown on the left (5% load) and the relative ratio of each form of RNAP II in SCP1 immunoprecipitates to that in lysates is given. The data shown is representative of three experiments.

[0049] **Figure 6. Effects of Phosphatase-active and Phosphatase-inactive SCP1 on gene expression.**

A. Effects of targeted SCP1 261 on reporter gene expression. Gal 4-DNA binding domain-SCP1 fusion protein expression plasmids were cotransfected in HEK293 cells with a Gal 4-TKLuc reporter plasmid and luciferase gene expression quantitated. In all panels, results of triplicate transfections are shown +/- SD. Data is expressed as fold-activation experimental/control).

B. Effects of SCP1 261 and mutant SCP1 261 on basal promoter activity. The indicated reporter plasmids were cotransfected with SCP1 or phosphatase-inactive SCP1.

C. Differing effects of SCP1 261 and phosphatase-inactive SCP1 261 on Gal 4-VP16 stimulated gene expression. The indicated amounts of SCP1 or mutant SCP1 expression plasmids were cotransfected in HEK293 cells along with Gal 4-VP16 expression and Gal 4-TK-Luc reporter plasmids and luciferase activity was quantitated.

D. Effects of SCP1 261 and phosphatase-inactive SCP1 261 on ligand activated receptor activity. The indicated receptor

expression plasmids were cotransfected with their cognate promoter-reporter plasmids with or without SCP1 or mutant SCP1 expression plasmids. Cells were treated or untreated with receptor-specific ligands as described under ExperimentalProcedures."

E. Competitive effects of mutant SCP1 261 with SCP1 261. The indicated concentrations of SCP1 and mutant SCP1 expression plasmids were cotransfected with LMX1 and E47 expression plasmids and the rat insulin 1 promoter-luciferase reporter gene. Luciferase activity was measured as described.

SCP1 nucleic acid sequence on chromosome 2

ctggagcgcg gcaggaaccc ggcccggccc gcctcccagt ccgcctagcc gcgccggtcc
cagaagtggc gaaagccgca gccgagtcca ggtcacgccg aagccgttgc ccttttaagg
gggagccttg aaacggcgcc tgggttccat gtttgcatcc gcctcgcggg aaggaaactc
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What is claimed is

1. An isolated nucleic acid molecule selected from the group consisting of:

a) the cDNA deposited with ATCC as Accession Number BE300370;

b) the cDNA deposited with ATCC as Accession Number AL520011; and

c) the cDNA deposited with ATCC as Accession Number AL520463,

or a complement thereof.

2. The nucleic acid molecule of claim 1 further comprising vector nucleic acid sequences.

3. The nucleic acid molecule of claim 1 further comprising nucleic acid sequences encoding a heterologous polypeptide.

4. A host cell which contains the nucleic acid molecule of claim 1.

5. The host cell of claim 4 which is a mammalian host cell.

6. A non-human mammalian host cell containing the nucleic acid molecule of claim 1.

7. An isolated polypeptide selected from the group consisting of:

a) the polypeptide encoded by the cDNA insert deposited with ATCC as Accession Number BE300370;

b) the polypeptide encoded by the cDNA insert deposited with ATCC as Accession Number AL520011; and

c) the polypeptide encoded by the cDNA insert deposited with ATCC as Accession Number AL520463.

8. The polypeptide of claim 7 further comprising heterologous amino acid sequences.

9. The polypeptide of claim 7, wherein the polypeptide is a phosphatase.

10. The polypeptide of claim 9, wherein the phosphatase is a small C-terminal domain phosphatase (SCP) that dephosphorylates RNA polymerase II.

11. The polypeptide of claim 10, wherein the phosphatase

is small C-terminal phosphatase-1 (SCP1), small C-terminal phosphatase-2 (SCP2), or splice variant thereof.

12. The polypeptide of claim 9, wherein the phosphatase dephosphorylates serine 5 within the C-terminal domain of RNA polymnerase II.

13. An antibody which selectively binds to a polypeptide of claim 7.

14. A method to increase gene expression by administering a nucleic acid encoding a phosphatase inactive mutant of SCP or its gene product.

15. The method of claim 14, wherein administrating a nucleic acid encoding a phosphatase inactive mutant of SCP or its gene product upregulates gene transcription by RNA polymerase II.

16. A method to decrease gene expression by enhancing the expression of wildtype SCP, or administering a nucleic acid encoding wildtype SCP or phosphatase active mutants of SCP and their gene products.

17. The method of claim 16, wherein enhancing the expression of wildtype SCP, or administering a nucleic acid encoding wildtype SCP or phosphatase active mutants of SCP and their gene products down-regulates gene transcription by RNA polymerase II.

18. A method of modulating the activity of a polypeptide of claim 7 comprising contacting the polypeptide or a cell expressing the polypeptide with a compound which binds to the polypeptide in a sufficient concentration to modulate the activity of the polypeptide.

19. A method for identifying a compound which modulates the activity of a polypeptide of claim 7 comprising:

a) contacting a polypeptide of claim 8 with a test compound; and

b) determining the effect of the test compound on the activity of the polypeptide to thereby identify a compound which modulates the activity of the polypeptide.

Abstract

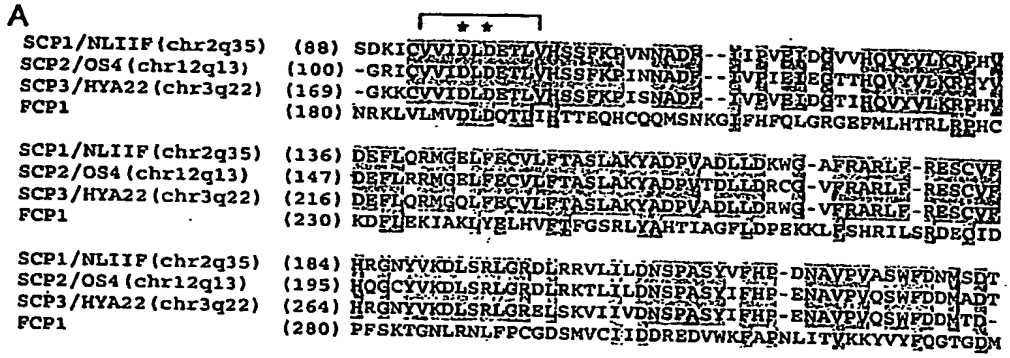
[0050] The transcription and processing of pre-mRNA in eukaryotic cells are regulated in part by reversible phosphorylation of the C-terminal domain of the largest RNA polymerase II subunit. The CTD phosphatase, FCP1, catalyzes the dephosphorylation of RNAP II and is thought to play a major role in polymerase recycling. This study describes a family of small CTD phosphatases (SCP) that preferentially catalyze the dephosphorylation of Ser 5 within the consensus repeat. The preferred substrate for SCP1 is RNAP II phosphorylated by TFIIH. Like FCP1, the activity of SCP1 is enhanced by the RAP74 subunit of TFIIF. Expression of SCP1 inhibits activated transcription from a number of promoters whereas a phosphatase-inactive mutant of SCP1 enhances transcription. Accordingly, SCP1 may play a role in the regulation of gene expression, possibly by controlling the transition from initiation/capping to processive transcript elongation.

PHOSPHATASE REGULATION OF NUCLEIC ACID TRANSCRIPTION

Gordon N. Gill et al.

15670-003P01

A



B

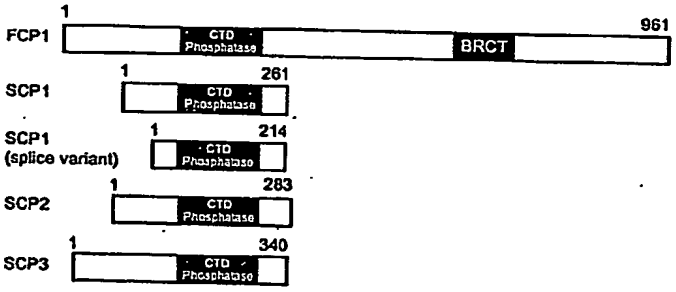


FIG. 1

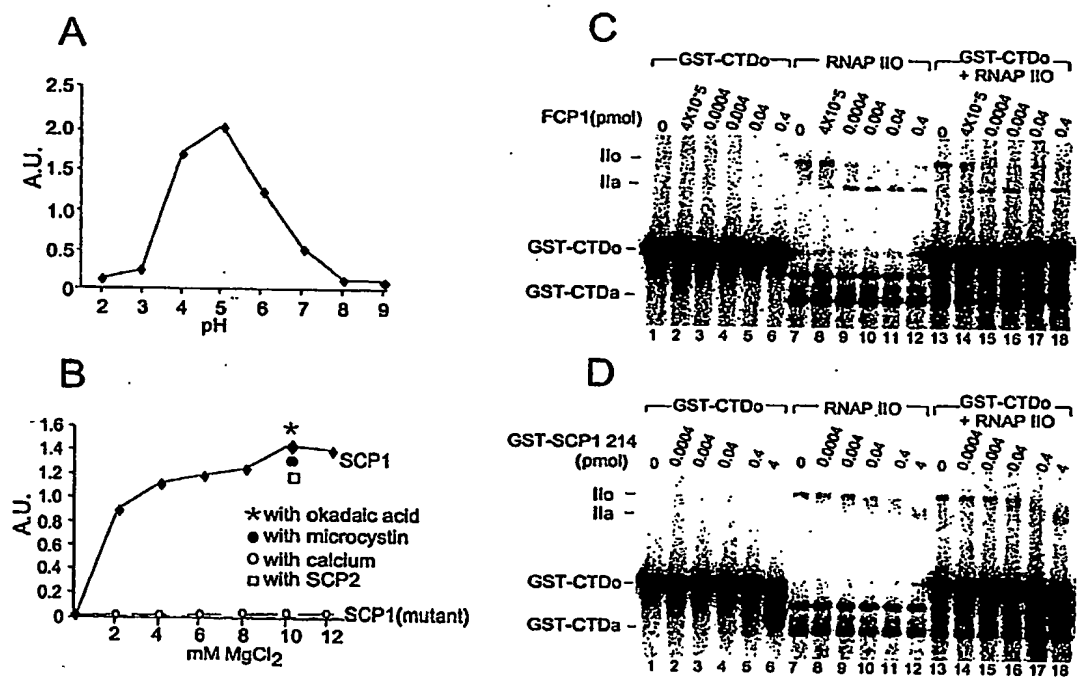

A
A.U.
pH
B
A.U.
SCP1
*with okadaic acid
● with microcystin
o with calcium
□ with SCP2
SCP1(mutant)
mM MgCl2
C
GST-CTDo
RNAP IIO
GST-CTDo + RNAP IIO
FCP1(pmol)
IIo
IIa
GST-CTDo
GST-CTDa
D
GST-SCP1 214 (pmol)

FIG. 2

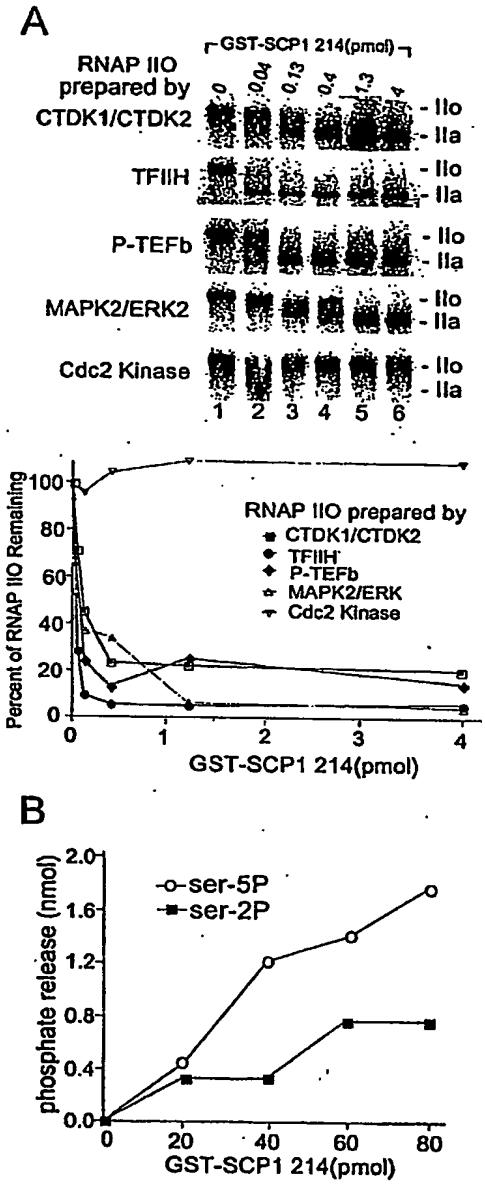

A
GST-SCP1 214(pmol)
0
0.04
0.13
0.4
1.3
4
RNAP IIO prepared by
CTDK1/CTDK2
TFIIH
P-TEFb
MAPK2/ERK2
Cdc2 Kinase
IIo
IIa
1 2 3 4 5 6
Percent of RNAP IIO Remaining
RNAP IIO prepared by
CTDK1/CTDK2
TFIIH
P-TEFb
MAPK2/ERK
Cdc2 Kinase
GST-SCP1 214(pmol)
B
phosphate release (nmol)
ser-5P
ser-2P
GST-SCP1 214(pmol)

FIG. 3

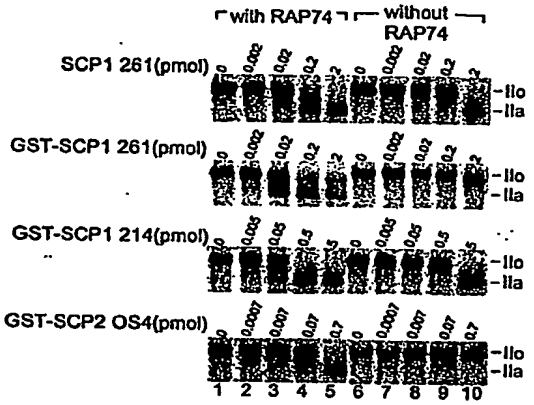

with RAP74
without RAP74
SCP1 261(pmol)
GST-SCP1 261(pmol)
GST-SCP1 214(pmol)
GST-SCP2 OS4(pmol)
IIo
IIa
1 2 3 4 5 6 7 8 9 10

FIG. 4

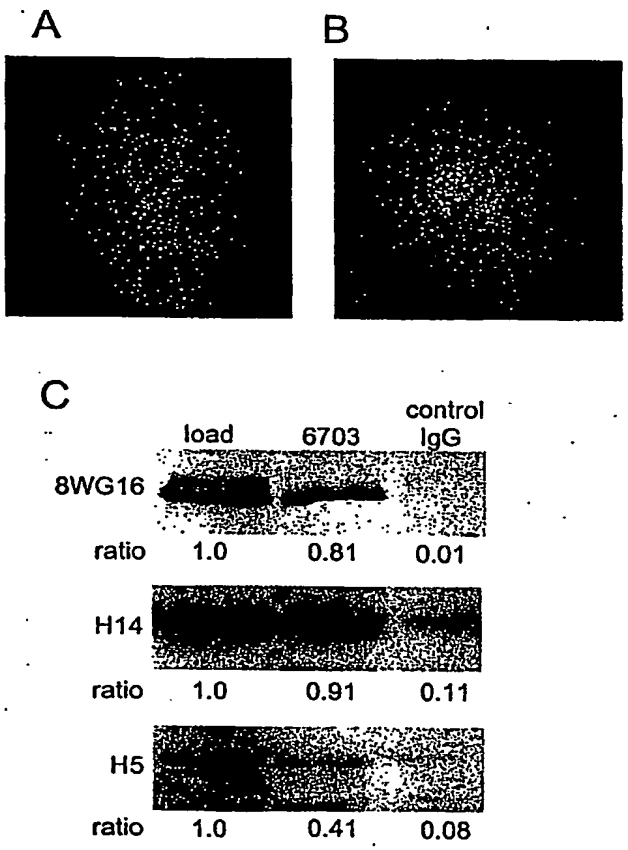

A
B
C
load
6703
control IgG
8WG16
ratio 1.0 0.81 0.01
H14
ratio 1.0 0.91 0.11
H5
ratio 1.0 0.41 0.08

FIG. 5

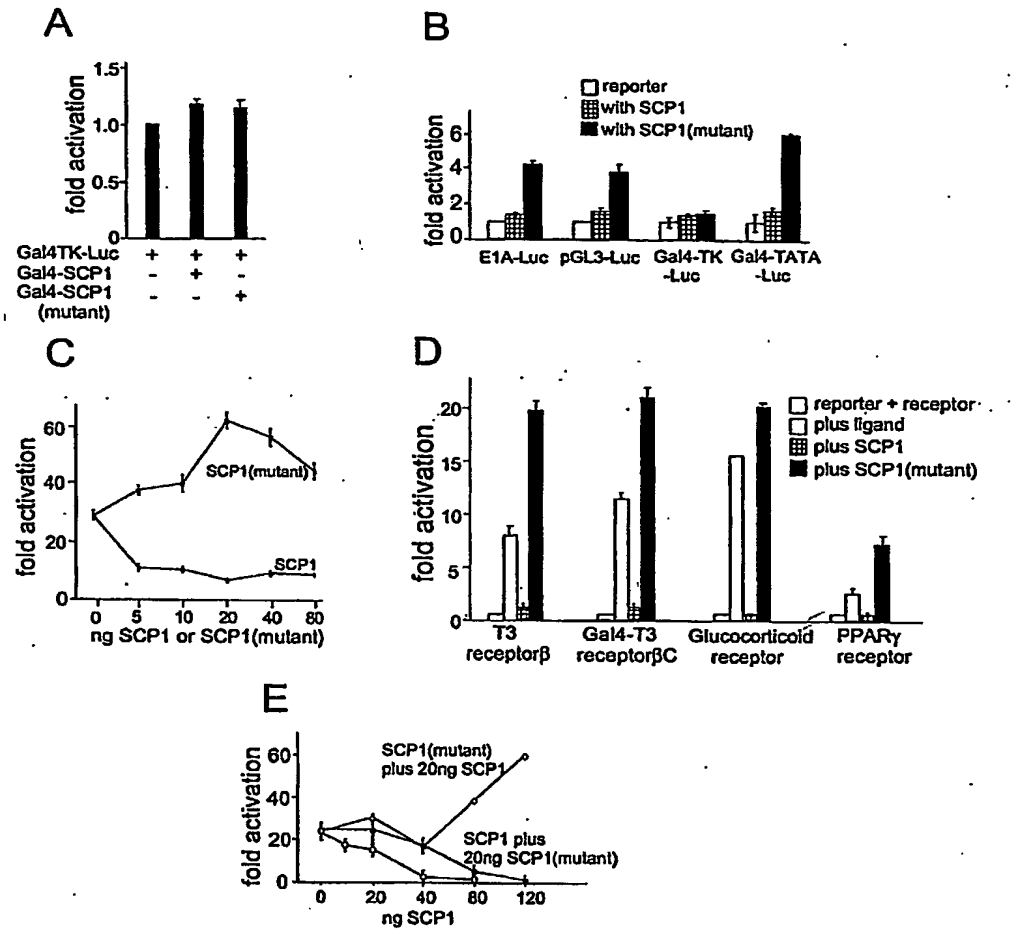

A
fold activation
1.5
1.0
0.5
0
Gal4TK-Luc + + +
Gal4-SCP1 - + -
Gal4-SCP1 (mutant) - - +
B
reporter
with SCP1
with SCP1(mutant)
fold activation
6
4
2
0
E1A-Luc
pGL3-Luc
Gal4-TK -Luc
Gal4-TATA -Luc
C
fold activation
60
40
20
0
SCP1(mutant)
SCP1
0 5 10 20 40 80
ng SCP1 or SCP1(mutant)
D
fold activation
20
15
10
5
0
reporter + receptor
plus ligand
plus SCP1
plus SCP1(mutant)
T3 receptorβ
Gal4-T3 receptorβC
Glucocorticoid receptor
PPARγ receptor
E
fold activation
60
40
20
0
SCP1(mutant) plus 20ng SCP1
SCP1 plus 20ng SCP1(mutant)
0 20 40 80 120
ng SCP1

FIG. 6